

# **Gingipains impair attachment of epithelial cell to dental titanium abutment surfaces**

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## **Abstract**

The study investigated in-vitro the effect of *Porphyromonas gingivalis* and its cysteine proteases (gingipains) on epithelial cell adhesion to titanium-zirconium alloy surfaces. Titanium-zirconium discs with a standard machined (M) or chemically modified hydrophilic surface (modM) were coated with lamin-5 and incubated with telomerase-inactivated gingival keratinocytes (TIGK). Three *P. gingivalis* strains or gingipains were either added simultaneously with TIGK or after TIGK cells were already attached to the disks. Adhered TIGK cells were counted at 24 hours. All *P. gingivalis* strains clearly inhibited adhesion of TIGK cells to M and modM surfaces. Compared with bacteria/gingipain-free TIGK cell cultures, the number of attached TIGK cells was reduced by about 80% and 60% when *P. gingivalis* was added simultaneously or after TIGK cells were already attached to the disks (each  $p < 0.01$ ), respectively. Counts of attached cells were similarly reduced when only gingipains were used. Adhesion molecules of TIGK cells, in particular E-cadherin, were cleaved by *P. gingivalis*. In conclusion, *P. gingivalis* and gingipains interfere with the adhesion of epithelial cells to titanium-zirconium alloy surfaces by cleaving adhesion molecules, while a chemically modified hydrophilic titanium-zirconium alloy surface did not yield any protection.

**Key Words:** epithelial cells, *Porphyromonas gingivalis*, titanium, dental implants

**Running heads:** Gingipains impair attachment of epithelial cell to titanium

## INTRODUCTION

Peri-implant diseases are becoming increasingly important. In a recent systematic review, the weighted mean prevalence of peri-implant mucositis and peri-implantitis among individuals with implants was found to be 43% and 22%, respectively.<sup>1</sup> Bacteria associated with periodontitis, such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* are also found more often in peri-implant lesions than in healthy controls.<sup>2-4</sup> In this context, in patients with a history of gingivitis or periodontitis, installed implant abutments are colonized by *P. gingivalis*, *T. forsythia*, *T. denticola*, *Fusobacterium nucleatum* and *Prevotella intermedia* latest one week after placement.<sup>5</sup>

*P. gingivalis*, a gram-negative anaerobe bacterium, is postulated to be one of the key bacteria associated with the development of periodontitis<sup>6</sup> and is also identified in diseased sites at implants.<sup>7</sup> *P. gingivalis* has a large arsenal of virulence factors including lipopolysaccharide, capsular material, fimbriae and proteases.<sup>8</sup> Among the different proteolytic enzymes secreted by *P. gingivalis*, the cysteine proteases, referred to as gingipains, are the most important.<sup>9</sup> Two genes (*rgpA* and *rgpB*) encode arginine-specific gingipains (HRgpA and RgpB) while a lysine-specific enzyme is a product of a single gene (*kgp*).<sup>10</sup> Acting alone, or in concert, gingipains are able to impair the immune response, as well as degrade the extra-cellular matrix and bioactive peptides.<sup>9,11</sup> *P. gingivalis* was localized in periodontal pocket epithelium, HRgpA was detected intracellularly.<sup>12</sup> Gingipains are able to degrade molecules that are essential in the integrity of epithelium.<sup>13,14</sup>

In this context, the peri-implant mucosa presents a well-keratinized and non-keratinized epithelium facing the oral cavity and the sulcus, respectively.<sup>15</sup> In particular, the peri-implant sulcular epithelium is moderately cohesive due to low abundance of fibrin threads.<sup>16</sup> It is thus considered that the peri-implant mucosa may be relatively vulnerable to infection. However, if an implant or an abutment has surface properties enhancing peri-implant soft tissue adhesion, including a more stable, cohesive, epithelial seal with better barrier function against microbial colonization it may exert a protective function against peri-implant diseases.

Thus the aim of this in-vitro study was to investigate the effect of *P. gingivalis* and its gingipains on epithelial cell adhesion to titanium-zirconium alloy surfaces, with or without hydrophilic modification.

## **MATERIAL AND METHODS**

### **Epithelial cells**

The telomerase-inactivated gingival keratinocytes (TIGK) cells <sup>17</sup>(provided by R. Lamont (University of Louisville, KY, USA) were maintained in cell cultivation media (Keratinocyte Growth Medium, KGM-Gold, Lonza, Basel, Switzerland). Confluent monolayers of epithelial cells were detached by trypsin / EDTA and the amount of epithelial cells was adjusted to about  $10^5$  per 1 ml of cell cultivation media.

### **Microorganisms and gingipains**

Experiments included three *P. gingivalis* strains (type strain ATCC 33277 and two well characterized clinical isolates J374-1 and M5-1-2) and purified gingipains (HRgpA and RgpB and Kgp). The M5-1-2 strain has a large slime capsule, whereas the highly fimbriated J374-1 strain forms rough colonies. Gingipains had been purified from culture medium of *P. gingivalis* strain H66 as described before.<sup>18</sup> Gingipains were kept frozen and were activated by an activation buffer containing cysteine hydrochloride before addition. Bacteria were stored at -80°C in a low passage. They were cultivated at Schaedler agar plates and passaged three or four times at 37°C with an anaerobic atmosphere before using in experiments. Bacteria were suspended in cell cultivation media to a concentration of about  $2 \times 10^6$ /ml.

### **Experimental disks**

Experimental disks (diameter 5 mm, height 1 mm) with a standardized machined surface (M, Ra 0.13-0.16  $\mu$ m) consisted of about 85% titanium and 15% zirconium (Roxolid®), Institute

Straumann AG, Basel, Switzerland). Additionally, in the cell adhesion experiments disks with a chemically modified hydrophilic surface of similar roughness (modM, Ra 0.13-0.16 µm) were included (ESTA®<sup>®</sup>, Institute Straumann AG).

### **Impact of *P. gingivalis* strains on epithelial cell attachment**

#### *Simultaneous application of epithelial cells and *P. gingivalis* strains*

Experiments were performed in 48-well plates. First, M disks and modM disks were covered with 25% of inactivated human serum, a major constituent of peri-implant sulcular fluid, and 0.1 µg/ml laminin-5 overnight at room temperature. Thereafter, bacterial suspension was mixed with the equal volume of TIGK cell suspension (ratio bacteria to TIGK cells 20 : 1) and added to the disks. Each disc was immersed in 1 ml of this suspension in a 48-well-plate. Plates were incubated with 5% CO<sub>2</sub> at 37°C for 24 h. Thereafter, adherent TIGK cells were fixed with methanol for 1 min, washed with phosphate buffered saline (PBS), stained with DAPI for 5 min and finally washed with PBS. Subsequently they were counted after visualization and capturing with the help of a fluorescent microscope (Olympus BX51, Tokyo, Japan; [Fig. 1](#)).

#### *Infection of epithelial cells being already attached to titanium surfaces with *P. gingivalis* strains*

In a second series of experiments, M and modM disks were coated as described before and TIGK cells were added alone. After 24 h of incubation, the cell cultivation media was removed and a bacterial suspension in cell cultivation media was added. Incubation was continued to for 24 h before cells were counted.

### **Impact of gingipains on epithelial cell adhesion**

The above series of experiments were then repeated with gingipains instead of the bacteria. Epithelial cells and activated gingipains in three concentrations (10 nM, 100 nM, 1  $\mu$ M) were used, based on the reported levels of gingipains in gingival crevicular fluid.<sup>19,20</sup>

### **mRNA expression of gingipains in *P. gingivalis* strains in contact with titanium-zirconium surfaces**

In these experiments only M disks were used. Gingipain expression (mRNA) at 1 h, 6 h and 24 h after addition of the bacteria to the TIGK with and without M disks, was compared with that of bacteria culture only. As before, bacteria were added simultaneously with TIGK or 24 h after TIGK were allowed to attach to M disks.

RNA was extracted by using the innuPREP RNA Mini Kit (Analytic Jena, Jena, Germany) and cDNA was generated from 100 ng total RNA by using the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturers' instructions. Thereafter, real-time PCR using GoTaq® qPCR Master Mix (Promega) with respective primers<sup>21</sup> was performed according to the manufacturer's recommendations. Quantification was made related to the 16S RNA gene expression of *P. gingivalis*.

### **Cleavage of adhesion molecules**

A potential cleavage and degradation of laminin-5, integrin  $\alpha$ 3, integrin  $\alpha$ 6, and E-cadherin was determined by a Western blot technique.

The number of adhered TIGK cells on the titanium-zirconia disks was not sufficient for reliable detection of potentially cleavage proteins by using Western blot. Thus, TIGK cells were cultured on 6-well plates for 24 h, cell cultivation medium was removed and cells were cultured

with *P. gingivalis* ATCC 33277 (20 bacterial cell per each TIKG cell) in the cultivation media for additional 24 h.

To visualize protein degradation, aliquots of cell cultures and bacterial suspension were resolved by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis. Cell cultures in the absence of *P. gingivalis* or gingipains were used as controls. To avoid protein degradation during sample preparation, the samples were treated with 0.05 mM D-Phe-Phe-Arg-chloromethyl ketone (Bachem AG, Bubendorf, Switzerland), boiled in non-reducing SDS treatment buffer and then re-boiled under reducing conditions before running on 10% SDS–Tricine polyacrylamide gels. Thereafter resolved proteins were blotted onto nitrocellulose membranes. Non-specific binding was blocked by incubation with TBST (20 mM Tris, pH 7.5, 0.5 M NaCl, 0.05% Tween 20) containing 5% nonfat dried milk or bovine serum albumin (BSA) for 1 h at room temperature. Then proteins of interest and their fragments were detected by incubation of the membranes overnight with monoclonal antibodies (mAbs) specific for the molecules (laminin-5: batch ab78286, abcam, Cambridge, UK; integrin  $\alpha$ 3: batch NBP1-97732, Novus Biologicals, Littleton, CO, USA; integrin  $\alpha$ 6: batch ab194969, Abcam; E-cadherin: batch ab76055, Abcam) in 1% BSA/T-TBS. After extensive rinsing with TBS-T, the immunoblots were incubated with conjugated secondary antibodies (Goat Anti Mouse-AP, batch 6790, Abcam) in 1% BSA/TBS-T. The blots were developed using a chemiluminescent substrate and immunoreactive bands were visualized by using a chemiluminescent blot scanner (Imager CHEMI premium; VWR, Radnor, PA, USA).

### **Statistical analysis**

In general, eight independent results were analyzed with ANOVA followed by post-hoc Bonferroni. Student's t-test for independent samples compared M vs. modM surfaces. SPSS 24.0 (IBM, Chicago, IL, USA) was used.

## RESULTS

### Impact of *P. gingivalis* strains on epithelial cell attachment

#### *Simultaneous application of epithelial cells and P. gingivalis strains*

All tested *P. gingivalis* strains clearly inhibited TIGK cell adhesion to disks with M surfaces coated with laminin-5. The number of attached cells after 24 h of incubation was about one fifth in comparison with those not being exposed to *P. gingivalis* (each  $p < 0.01$ ). There were no significant differences among the strains (Figure 2A).

The modM surface promoted the attachment of TIGK cells after 24 h ( $p = 0.024$ ) when compared to the M surface. Independently of which strain was used, the numbers of attached TIGK cells dropped down to the same level as for the M surfaces (each  $p < 0.001$ ) in comparison with controls not exposed to *P. gingivalis*, Figure 2C).

#### *Infection of epithelial cells being already attached to titanium surfaces with P. gingivalis strains*

If TIGK cells were allowed first to adhere to the disks then treated with *P. gingivalis*, the total time of incubation was 48 h. The numbers of cells on both surfaces increased after 48 h in comparison to 24 h but the difference reached significance ( $p = 0.005$ ) only in the case of M surfaces. Regardless which *P. gingivalis* strains were added, about 70-80% less TIGK cells ( $p < 0.001$ ) were found attached to M and modM surfaces after 48 h, compared to non-infected disks (Figure 2B).

### Impact of gingipains on epithelial cell adhesion

When gingipains in three concentrations (1 nM, 10 nM, 100 nM) were used together with TIGK cells, the number of adherent cells was reduced to the level of 0.5% - 50% (each  $p < 0.001$ ) in



comparison to the number TIGK cells on M surfaces without gingipains. A clear concentration dependency was found, and the lowest number of cells was counted when 100 nM HRgpA was used (vs. 1 nM  $p<0.001$ , vs. 10 nM  $p=0.034$ ; Figure 3A).

When gingipains were added 24 h after the TIGK cells, results were similar, with the number of attached TIGK cells at about 1.5% - 45% of the number of cells observed at non-exposed controls. All differences for each gingipain and used concentration were statistically significant ( $p<0.001$ ). Concentration dependency was found for 100 nM of HRgpA vs. 1 nM ( $p=0.001$ ) and for Kgp vs. 10 nM; ( $p=0.026$ ; Figure 3B).

In the series using modM surfaces, gingipains were applied only at 100 nM concentration. The numbers of attached TIGK cells decreased to 0.3% - 13.3% (each  $p<0.001$ ) when gingipains were used simultaneously with the cells, and to 0.2% - 56.5% (each  $p<0.001$ ) when gingipains were added at adherent cells. Lowest numbers were counted for HRgpA and the highest for RgpB, irrespective of time of application (Figure 3C, 3D).

### **mRNA expression of gingipains in *P. gingivalis* strains in contact with titanium surfaces**

In each sample, expression of gingipains was related to those of 16S RNA.

In *P. gingivalis* only cultures, a decrease in expression of all gingipains over time compared with the levels at 1 h was observed; at 6 h the decrease was by 12-14-fold (not statistically significant), while at 24 h there was a decline by about 50-fold for all gingipains (*rgpA*:  $p=0.030$ , *rgpB*:  $p=0.001$ , *kgp*:  $p=0.001$ ).

When *P. gingivalis* was added 24 h after the TIGK cells to M surfaces *rgpA* expression was higher by trend (4.5-fold) related to bacteria only at 1h. At 6 h and 24 h, *rgpB* expression was about 10-fold and 50-fold related to bacteria only at the respective times when bacteria were cultured simultaneously with TIGK cells, irrespective whether M disks were used or not (6 h:

bacteria 24 h after TIGK to M surfaces  $p=0.026$ , 24 h: bacteria in contact with TIGK  $p=0.006$ , simultaneous addition of bacteria and TIGK to M disks  $p=0.014$ , bacteria 24 h after TIGK to M disks  $p=0.013$ ).

At 1 h and 6 h, *rgpB* expression was highest when bacteria were added 24 h after TIGK to M disks (difference to bacteria only at 1 h  $p=0.010$ , at 6 h  $p<0.001$ ; to simultaneous addition of bacteria and TIGK to M disks at 1 h  $p=0.013$ , at 6 h  $p=0.005$ ). At 24 h, expression was about 45-fold when bacteria were in contact with TIGK without M disks or added 24 h after TIGK to M disks related to bacteria only ( $p=0.008$ ,  $p=0.006$ ).

At all three times, *kgp* expression was statistically significantly higher when bacteria were in contact with TIGK without M disks or added 24 h after TIGK to M disks related to bacteria only, at 1h expression was 7.5-fold ( $p=0.010$ ) and 12-fold ( $p=0.001$ ), at 6 h expression was about 15-fold each ( $p=0.001$ ;  $p<0.001$ ) and at 24 h 50-fold each (both  $p<0.001$ ). At 24 h, *kgp* expression was also higher when bacteria and TIGK were added simultaneously to M disks related to bacteria only ( $p=0.003$ ) (Figure 4).

### **Cleavage of adhesion molecules**

To get a relevant number of TIGK cells for immunoblotting, plastic surfaces were used for attachment. Exposure of cells to *P. gingivalis* confirmed cleavage and degradation of their adhesion molecules. Additional bands resembling lower molecular weight molecules were visible for all four tested molecules (integrin  $\alpha 6$ , integrin  $\alpha 3$ , E-cadherin and laminin 5) when bacteria were added to TIGK cells (Figure 5).

## **DISCUSSION**

This in-vitro study aimed to investigate the effect of *P. gingivalis* and its cysteine proteases (gingipains) on epithelial cell adhesion to a titanium-zirconia alloy surface. Two different clinical scenarios were simulated herein: a) infection during implant/abutment placement, i.e. when TIGK cells and bacteria or gingipains were used simultaneously, or b) infection at a later time-point, i.e., when bacteria or gingipains were added 24 h after TIGK cell culturing.

To mimic an in vivo situation titanium-zirconia disks were covered with laminin-5, a receptor for epithelial cells, and with human serum, a major constituent of peri-implant sulcular fluid. Laminin-5 is known to function as a binding molecule for epithelial cells via  $\alpha 6\beta 4$  and  $\alpha 3\beta 1$  integrins.<sup>22</sup> On this note, the coating of titanium surfaces with laminin-5 has been shown to promote epithelial cell adhesion.<sup>23</sup> It has been previously shown that *P. gingivalis* interfered with oral keratinocyte cell adhesion and spreading on plastic surfaces coated with laminin-5,<sup>22</sup> and that coating of titanium disks with laminin promoted adhesion of *P. gingivalis*.<sup>24</sup> Herein, we confirmed that *P. gingivalis* (the reference strain and the two included clinical isolates) clearly prevented epithelial cell adhesion on titanium-zirconia disks coated with laminin-5.

The virulence of *P. gingivalis* depends on its gingipains' expression.<sup>9</sup> In gingival crevicular fluid from deep periodontal pockets concentration of gingipains were found up to 1.5  $\mu\text{M}$  for Arg-gingipains<sup>20</sup> and 10 nM for Lys-gingipain<sup>19</sup>. No data are currently available from the clinic for dental implants. However, we have recently shown in vitro that, expression of gingipains was decreased when *P. gingivalis* attached to titanium surfaces compared with dentin surfaces.<sup>25</sup> In the present study, a decrease of gingipains expression over time was confirmed for bacterial suspension in cell culture medium. However, gingipain expression was higher when the bacteria were in contact with epithelial cells independent of the presence of titanium-zirconia surfaces compared with bacterial suspensions alone, suggesting that epithelial cells stimulate somehow gingipain expression. This is in line with a previous report about *rgpB* upregulation when *P. gingivalis* was contact with immortalized epithelial human cells compared to pure bacterial cultures.<sup>26</sup> In the present study, a very low adhesion of TIGK cells to titanium-zirconia surfaces and a potent detachment of TIGK cells from the disks was observed when purified

gingipains were used, compared with the use of *P. gingivalis* strains. This underlines the role of gingipains as essential virulence factors.

Gingipains have been shown to degrade a number of cell components e.g. N-cadherin and thus induce detachment and apoptosis of human epithelial KB-cells.<sup>27</sup> Per se, gene expression of certain integrins such as  $\alpha 6$ , integrin  $\beta 4$  have been found to be less pronounced on titanium than on plastic surfaces.<sup>28</sup> A cleavage of integrins may further decrease their functional numbers. Herein, E-cadherin was clearly degraded into fragments when TIGK cells were infected with *P. gingivalis*. This is in contrast to another study<sup>13</sup> using a different immortalized cell line and shorter times of infection compared to the present study. However, using bacterial strains treated or untreated with cysteine protease inhibitors and purified gingipains, degradation of E-cadherin by all three gingipains was shown, with Kgp being most active.<sup>14</sup> Gingipain ability of cleaving cell adhesion molecules might also be of importance and it has been shown that the gingipains RgpA and Kgp have a strong binding affinity to laminin.<sup>29</sup> Cleavage of laminin-5 has indeed been shown to occur in vivo in periodontal disease<sup>30</sup> and also in mucositis and peri-implantitis sites.<sup>31</sup> Nevertheless, this might be not only related to bacterial protease activity, since host-derived proteases e.g. neutrophil elastase, are highly efficient in cleaving laminin-5.<sup>32</sup>

From a clinical perspective, impairment of epithelial cell adhesion to abutment surfaces might be of relevance regarding the establishment of an effective biological barrier. For example, *P. gingivalis* has been detected in the interior aspects of implants about 25 days after placement of healing abutments, due to contamination via the implant-abutment microgap<sup>23</sup>, which in turn may contribute to development of peri-implant biological complications later on. Fast establishment of an effective biological barrier through accelerated epithelial cell adhesion, on the other hand, might prohibit micro-gap contamination. Results from a study in dogs suggest that surface hydrophilicity rather than microtopography influences soft tissue integration.<sup>33</sup> In vitro it has been shown that a hydrophilic surface increases attachment of epithelial cells in comparison with a hydrophobic one, whereas a rougher surface may have a lower adhesive

propensity for epithelial cells compared to a smoother one.<sup>28</sup> Results of a proof-of-concept study in humans have suggested that this hydrophilic surface may have potential to enhance soft tissue adhesion.<sup>34</sup> Further, it has been demonstrated that smooth titanium surfaces show slower microbial colonization compared to rougher surfaces. Thus, in the present study, a modified smooth, hydrophilic implant surface (ESTA) was tested. It was thus hypothesized that the ESTA surface may on the one hand enhance TIGK cell adhesion and on the other hand limit *P. gingivalis* adhesion. However, modM was not shown to inhibit the negative influence by *P. gingivalis* and its gingipains, as no significant differences were observed between the M and the modM surface in any of the tests performed herein.

*P. gingivalis* may also interfere with epithelial cells via other cells, Kgp induces interleukin-31 in mast cells which possibly increases permeability of the epithelial barrier.<sup>35</sup> Except for *P. gingivalis*, also other bacterial might play a role in disturbing epithelial attachment to the titanium surface. Recently we have shown that the presence of *P. gingivalis* but also of *T. forsythia* and *T. denticola* were associated with disease severity in the periodontal and peri-implant tissues; the level of expression of gingipains was associated with those of certain *T. forsythia* proteases around teeth but not implants.<sup>4</sup> *T. forsythia* synthesizes a sialidase which is involved in epithelial cell binding and invasion.<sup>36</sup> An outer membrane protein of *T. denticola* was described to mediate adhesion to epithelial cells.<sup>37</sup>

In further research might be of interest to verify the role of other bacteria including their virulence factors enzymes in impairment of epithelial cell adhesion to abutment surfaces as an essential step in initiation of peri-implant diseases.

## CONCLUSION

*P. gingivalis* and gingipains interfere with the adhesion of epithelial cells to titanium-zirconium alloy surfaces by cleaving adhesion molecules, while a chemically modified hydrophilic titanium-zirconium alloy surface did not yield any protection.

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## Figures legends

**Figure 1.** Fluorescent microscopy photographs of gingival epithelial cells attached to titanium-zirconium alloy with standard machined (A, B, D, E, F) or a chemically modified hydrophilic (C) surface without (A; control) or in the presence of *Porphyromonas gingivalis* ATCC 33277 (B, C) or 10 nM of gingipains (HRgpA (D), RgpB (E), Kgp (F)) after 24 h of co-culture

**Figure 2.** Adhesion of gingival epithelial cells without (control) or in the presence of *P. gingivalis* strains to titanium-zirconium alloy with a standard machined (M) and a chemically modified hydrophilic surface (modM)

A) Bacteria (*Porphyromonas gingivalis* ATCC 33277, *P. gingivalis* J374-1 or *P. gingivalis* M5-1-2) and cells were added simultaneously and incubated together for 24 h

B) Cells were allowed to adhere for 24 h, before bacteria (*Porphyromonas gingivalis* ATCC 33277, *P. gingivalis* J374-1 or *P. gingivalis* M5-1-2) were added and incubated together for 24 h

**Figure 3.** Adhesion of gingival epithelial cells to titanium-zirconium alloy with standard machined (M; A, B) or a chemically modified hydrophilic (modM; C, D) surface without (control) or in the presence of different concentrations (1 nM, 10 nM, 100 nM; modM only 100 nM) of gingipains (HRgpA, RgpB, Kgp;) after 24 h of co-culture (A, C) or when cells were allowed to adhere for 24 h, before gingipains were added for 24 h (B, D).

**Figure 4.** mRNA expression of *rgpA* (A), *rgpB* (B) and *kgB* (C) in *P. gingivalis* strains (ATCC 33277, M5-1-2, J374-1) in bacterial controls (con bacterial only), bacteria in contact with epithelial cells (con bacteria cells) as well when bacteria were added together with epithelial

cells to titanium (simultaneous) or when bacteria were added 24 h after epithelial cells were allowed to adhere to titanium related to bacterial culture at 1 h

Means of three strains (each determined in independent quadruplicates) are presented. mRNA expression of gingipains was adjusted to those of 16S RNA each.

**Figure 5.** Blots on integrin  $\alpha 6$  (A), integrin  $\alpha 3$  (B), E-cadherin (C) and laminin 5 (D) of epithelial cells (c), epithelial cells exposed 24 h to *P. gingivalis* ATCC 33277 (c+b) and *P. gingivalis* ATCC 33277 only (b)



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